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Evaluation of the antidiabetic and antidyslipidemic activities of 80% methanolic leaf and seed extracts of *Calpurnia aurea* (Ait.) Benth. (Fabaceae) in mice

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This is to certify that the thesis prepared by Mr. Yaschilal Muche, entitled: "*Evaluation of the antidiabetic and antidyslipidemic activities of 80% methanolic leaf and seed extracts of Calpurnia aurea (Ait.) Benth. (Fabaceae) in mice"* and submitted in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Background: Currently diabetes mellitus is one of the largest global health emergencies. As the morbidity and mortality rate of diabetes is increasing worldwide, it is necessary to investigate medicinal plants for new antidiabetic drugs that can lead to effective and safe pharmacotherapy.

Objective: To evaluate the antidiabetic and antidyslipidemic activities of 80% methanolic leaf and seed extracts of *Calpurnia aurea* (Ait.) Benth. (Fabaceae) in mice.

Method: Blood glucose lowering activity of the three doses (100mg/kg, 200mg/kg and 400 mg/kg) of hydromethanolic leaf extract and three doses (2.75 mg/kg, 5.5 mg/kg and 11 mg/kg) of the hydromethanolic seed extract of *Calpurnia aurea* was studied in four animal models; normoglycemic mice, oral glucose loaded mice, single dose treated diabetic mice and repeated dose treated diabetic mice. Additionally, the effect of the extracts on body weight and serum lipid profile was studied in the repeated dose treated diabetic mice. Glibenclamide (5 mg/kg) was used as a standard drug in all animal models. Blood glucose level was measured using a glucose meter; whereas serum lipid levels were measured using an automated chemistry analyzer. Data were analyzed using one way analysis of variance followed by Tuckey's post hoc test.

Result: Hydromethanolic extract of *calpurnia aurea* seeds showed blood glucose lowering activity in all animal models, and it improves body weight loss and diabetic dyslipidemia in diabetic animals. But, hydromethanolic extract of *Calpurnia aurea* leaves lack significant blood glucose lowering activity in all animal models, and it didn't significantly improve the body weight loss and diabetic dyslipidemia of diabetic mice after 14 days of treatment.

Conclusion: This study revealed that hydromethanolic extract of *Calpurnia aurea* seeds has a significant antidiabetic and antidyslipidemic activity justifying the traditional use of the plant for diabetes mellitus. But, it was found that the leaves of the plant lack significant antidiabetic and antidyslipidemic activities.

Key words: Diabetes mellitus, *Calpurnia aurea*, Streptozotocin, Mice

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List of Abbreviations and Acronyms

ANOVA	Analysis of variance
BGL	Blood glucose level
DPP-4	Dipeptidyl peptidase-4
DM	Diabetes Mellitus
DW	Distilled water
FPG	Fasting Plasma Glucose
GDM	Gestational Diabetes Mellitus
GLC	Glibenclamide
HAART	Highly active antiretroviral therapy
HbA_{1c}	Glycated Hemoglobin
HNF	Hepatocyte nuclear factor
IDF	International Diabetes Federation
MODY	Maturity-Onset Diabetes of the Young
OECD	Organization for Economic Cooperation and Development
OGTT	Oral Glucose Tolerance Test
PKC	Protein kinase C
ROS	Reactive oxygen species
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
USD	United States Dollar
WHO	World Health Organization

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1 Introduction

1.1 Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from abnormalities in insulin secretion, insulin action, or both (1). It is one of four priority non-communicable public health problems targeted for action by world leaders (2). The chronic hyperglycemia of diabetes is associated with long-term microvascular complications affecting the eyes, kidneys and nerves, as well as an increased risk for cardiovascular disease (1, 3). Prediabetes is a term referring to impaired fasting glucose, impaired glucose tolerance or a glycated hemoglobin (HbA_{1C}) of 6.0% to 6.4%, each of which places individuals at high risk of developing diabetes and its complications (1).

1.1.1 Prevalence and global burden of diabetes mellitus

Globally, 422 million adults were living with diabetes in 2014. Over the past decade, diabetes prevalence has risen faster in developing countries than in developed countries (2). The global prevalence of DM is expected to rise to 552 million by 2030 (4) and 642 million by 2040 (5). The most important demographic change to diabetes prevalence across the world appears to be the increase in the proportion of people greater than 65 years of age (6).

Diabetes and its complications are major causes of early death in most countries. Approximately 5 million people aged between 20 and 79 years died from diabetes in 2015, equivalent to one death every six seconds. Additionally, DM accounted for 14.5% of global all-cause mortality among people in this age group (5).

Globally, 12% of the total health expenditure was spent on diabetes in 2015 (5). The global health expenditure on diabetes is estimated to be at least USD 376 billion in 2010 and it is expected to rise to USD 490 billion in 2030 (7) and USD 802 billion in 2040 (5).

In 2015, 14.2 million adults aged 20-79 have diabetes in Africa, representing a regional prevalence of 2.1 - 6.7%. Africa’s most populous countries have the highest numbers of people

with diabetes, South Africa (2.3 million), Democratic Republic of Congo (1.8 million), Nigeria (1.6 million) and Ethiopia (1.3 million) (5).

1.1.2 Classification and pathophysiology of diabetes mellitus

There are three main types of diabetes; type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), and gestational diabetes mellitus (GDM). Other less common types of diabetes are monogenic diabetes and secondary diabetes (5, 8).

1.1.2.1 Type 1 diabetes mellitus

Type 1 DM accounts for 5% to 10% of all DM cases. It is an autoimmune disease involving destruction of the insulin secreting β-cells of the pancreas which takes place over many years. Markers of the immune destruction of the beta-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2b. One and usually more of these autoantibodies are present in 85-90% of individuals when fasting hyperglycemia is initially detected (8). Autoimmune destruction of β-cells has multiple genetic predispositions and is also related to environmental factors that are still poorly defined (9). Type 1 DM commonly occurs in childhood and adolescence, but it can occur at any age, even in the 8^{th} and 9^{th} decades of life and it often develops suddenly (5).

1.1.2.2 Type 2 diabetes mellitus

Type 2 DM accounts for 90-95% of all diabetes cases (8). The pathophysiology of type 2 diabetes mellitus is characterized by peripheral insulin resistance and declining β-cell function, and both are influenced by genetic and environmental factors (10).

The primarily factor for insulin resistance in type 2 diabetes is post binding defects in insulin action, including reduced insulin receptor tyrosine kinase activity, insulin signal transduction abnormalities, decreased glucose transport, diminished glucose phosphorylation, and impaired glycogen synthase activity. Diminished insulin binding, when present, is modest and secondary to down-regulation of the insulin receptor by chronic hyperinsulinemia (11). In T2DM, the sensitivity of the beta-cell to glucose is impaired, and there is also a loss of responsiveness to other stimuli such as insulinotropic gastrointestinal hormones and neural signaling. This defect is compounded by a gradual loss of beta-cell mass over time, potentially related to toxic effects of

hyperglycemia (12). Chronic hyperglycemia causes toxic effects on structure and function of organs, including the pancreatic islet through multiple biochemical pathways which leads to the formation of reactive oxygen species that, in excess and over time, cause chronic oxidative stress, which in turn causes defective insulin gene expression and insulin secretion as well as increased apoptosis of beta cells (figure 1) (13, 14).

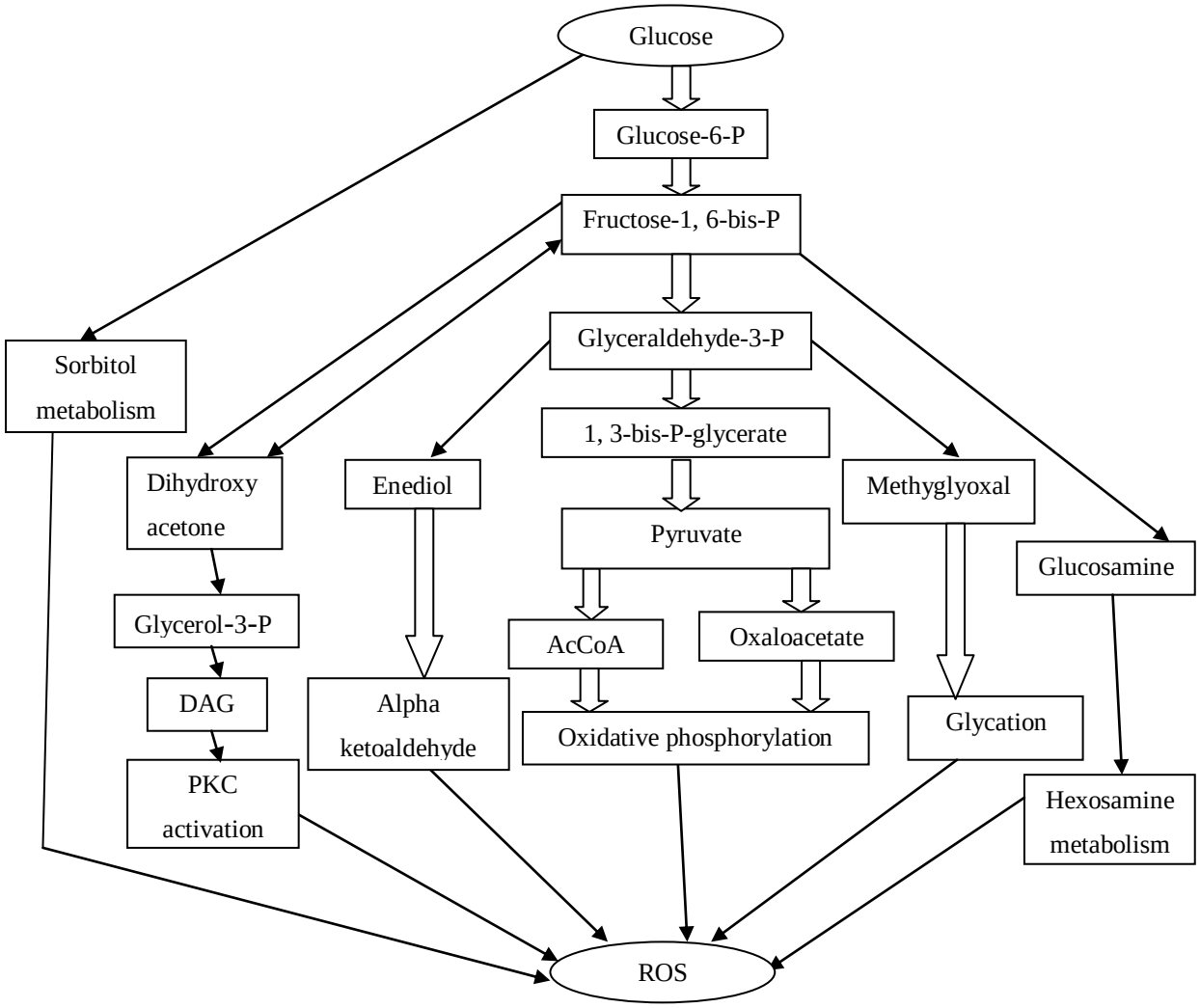

Figure 1: Six biochemical pathways along which glucose metabolism can form reactive oxygen species. DAG, diacylglycerol

1.1.2.3 Gestational diabetes

Gestational DM is defined as carbohydrate intolerance resulting in hyperglycemia of variable severity with onset or first recognition during pregnancy. The disorder affects 5% to 7% of all

pregnancies and its frequency is rising worldwide (15). Patients with GDM have a 30% to 50% chance of developing DM, usually type 2 DM (16).

Insulin resistance increases in normal pregnancy due to progressively rising levels of feto-placental hormones such as progesterone, cortisol, growth hormone, prolactin and human placental lactogen (15).

1.1.2.4 Other less common types of DM

Other less common types of diabetes include monogenic diabetes and secondary diabetes. Monogenic diabetes is the result of a genetic mutation. Examples of monogenic diabetes include Maturity-Onset Diabetes of the Young (MODY) and Neonatal Diabetes Mellitus. Secondary diabetes arises as a complication of other diseases, such as hormone disturbances (Cushing's disease or acromegaly) or diseases of the pancreas (5, 8).

Maturity-Onset Diabetes of the Young can result from mutations in at least six different genes, which were numbered according to the sequence of discovery. One of these encodes the glycolytic enzyme, glucokinase (MODY2), which is an important glucose sensor, whereas the others encode transcription factors: hepatocyte nuclear factor (HNF) 4α (MODY1), HNF-1α (MODY3), insulin promoter factor-1 (IPF1/Pdx-1; MODY4), HNF-1β (MODY5) and neurogenic differentiation factor 1 (NeuroD1; MODY6) (17, 18).

1.1.3 Risk factors for diabetes

The exact causes of type 1 diabetes are unknown. It is the result of a complex interaction between genes and environmental factors; though no specific environmental risk factors have been shown to cause a significant number of cases. The risk of type 2 diabetes is determined by interplay of genetic and metabolic factors. Overweight and obesity are the strongest risk factors for type 2 diabetes. Risk factors for GDM include age (the older a woman of reproductive age is, the higher her risk of GDM); overweight or obesity; excessive weight gain during pregnancy; a family history of diabetes; GDM during a previous pregnancy (2, 5).

1.1.4 Diabetes mellitus and dyslipidemia

Diabetes mellitus directly affect lipid levels (19). Diabetic patients tend to have higher serum levels of triglyceride (TG), lower high-density lipoprotein cholesterol (HDL-C), and similar

serum values for low-density lipoprotein cholesterol (LDL-C) but with high small dense LDL levels when compared with non-diabetic patients (20, 21). Hypertriglyceridemia is considered the dominant lipid abnormality and plays a pivotal role in determining the characteristic lipid profile of diabetic dyslipidemia (21). Insulin deficiency in diabetes reduces suppression of hormone sensitive lipase activity thereby increasing intracellular hydrolysis of TGs in the adipose tissue, consequently releasing free fatty acids (FFA) in the portal circulation. These FFA stimulate the assembly and secretion of very-low-density lipoprotein (VLDL) from the liver, resulting in excess circulating TG concentration (20, 21). Insulin deficiency and resistance is also associated with decreased lipolytic activity of lipoprotein lipase (LPL) which leads to decreased clearance of very low density lipoproteins (VLDLs) and chylomicrons (22).

1.1.5 Diagnosis of Diabetes Mellitus

Criteria for the diagnosis of diabetes includes; when $HbA_{1C} \geq 6.5\%$ (the test should be performed in a laboratory using a method that is National Glycohemoglobin Standardization Program certified and standardized to the Diabetes Control and Complications Trial assay), or fasting blood glucose ≥126 mg/dl (fasting is defined as no caloric intake for at least 8 h), or in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose $\geq$ 200 mg/dl (8).

Screening for GDM is done with a standard oral glucose tolerance test (OGTT). The 75 g OGTT is done in the morning after an overnight fast of at least 8 hours with plasma glucose measurements of fasting and at 1 and 2 hours following the oral glucose load. The diagnosis is confirmed when any of the following plasma glucose values are obtained: Fasting $\geq$ 92 mg/dl (5.1 mmol/L), 1 h $\geq$ 180 mg/dl (10.0 mmol/l) or 2 h $\geq$ 153 mg/dl (8.5 mmol/l) (8, 23, 24).

1.1.6 Treatment of diabetes mellitus

The primary goals of DM management are to reduce the risk for microvascular and macrovascular disease complications, to ameliorate symptoms, to reduce mortality, and to improve quality of life (24).

Insulin replacement therapy is the mainstay for patients with type 1 DM while diet, lifestyle modifications and oral hypoglycemic agents are considered the cornerstone for the management

of type 2 DM. Insulin is also important in type 2 DM when blood glucose levels cannot be controlled by diet, weight loss, exercise and oral medications (16). Oral hypoglycaemic agents include α-glucosidase inhibitors, biguanides, Insulin secretagogue (meglitinides and sulfonylureas), peroxisome proliferator-activated receptor γ agonists (thiazolidinediones or glitazones), Dipeptidyl peptidase-4 (DPP-4) inhibitors, dopamine agonists, bile acid sequestrants, and sodium-glucose cotransporter 2 inhibitors (25). The oral hypoglycemic agent metformin should be included in the therapy for all type 2 DM patients, if tolerated and not contraindicated, as it is the only oral antihyperglycemic medication proven to reduce the risk of total mortality, according to the United Kingdom Prospective Diabetes Study (26).

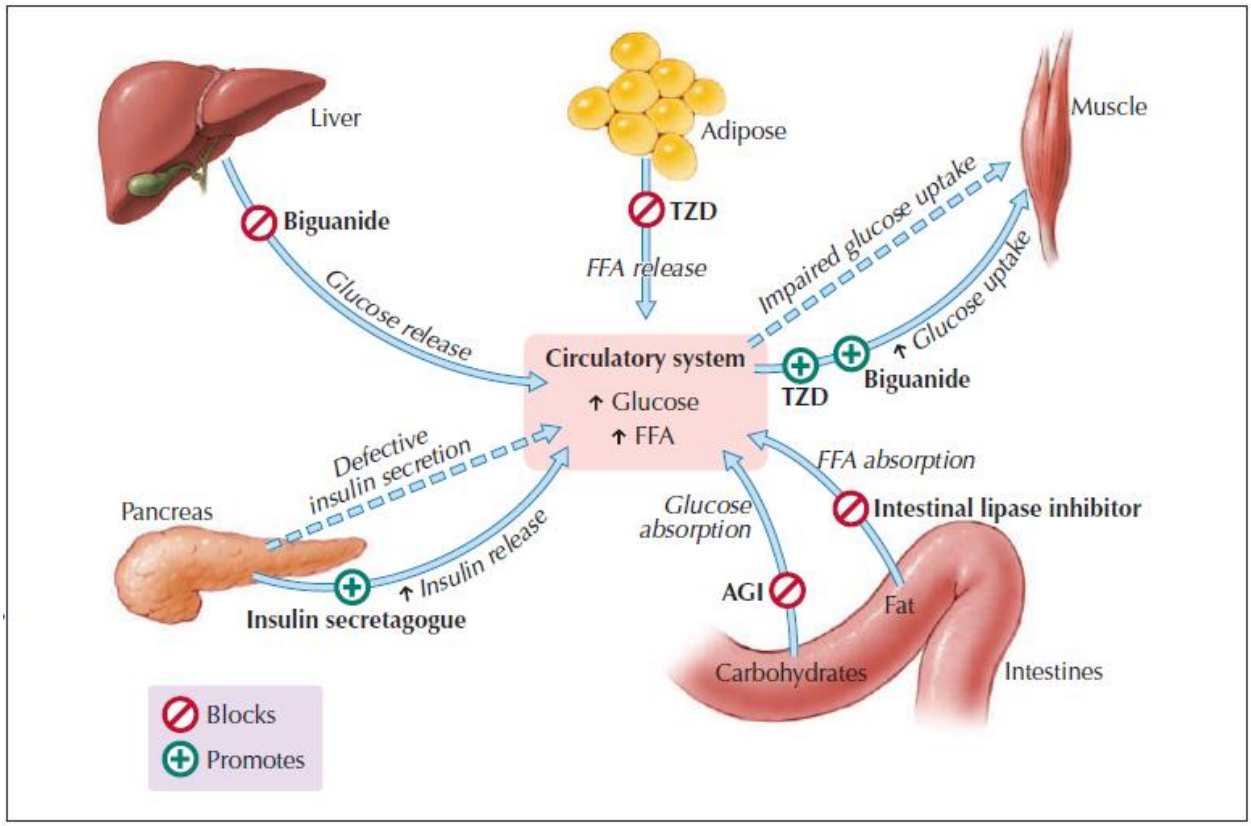

Figure 2: Major target organs and mechanism of action of oral hypoglycemic agents in type 2 diabetes mellitus. TZD = thiazolidinedione; FFA = free fatty acid; AGI = α-glucosidase inhibitor (27).

Dietary restrictions remain the mainstay of GDM management, and suitable physical exercise can help too. Rapid-acting insulin analogues (lispro and aspart) are novel treatments for improving metabolic control by reducing postprandial glycemia, while long-acting insulin

analogues need further study on the related safety issues before they can be prescribed. Numerous studies have found glyburide and metformin safe in GDM pregnancies, but more randomized controlled trials are needed in type 2 diabetic and GDM women, with a long-term follow-up of mother and child, to confirm these results (15, 25).

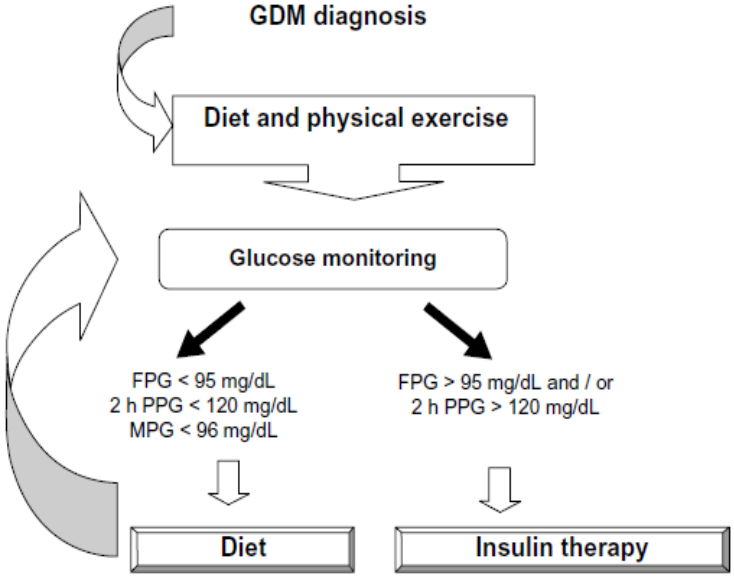

Figure 3: Management of gestational diabetes. FPG, fasting plasma glucose; 2 h PPG, 2 hour postprandial glucose; MPG, mean plasma glucose (15).

1.2 Medicinal plants and diabetes mellitus

Medicinal plants have been used in traditional health care systems since prehistoric times and are still the most important health care source for the vast majority of the population around the world (28). It is estimated that 70-80% of people worldwide rely on traditional herbal medicine to meet their primary health care needs. Globally, millions of people rely on medicinal plants not only for primary health care, but also for income generation and livelihood improvement (29).

Traditional herbal medicines play an important role in the management of diabetes mellitus. It was estimated that more than 1000 plant species are being used as folk medicine for diabetes (28). In recent years, herbal medicines have started to gain importance as a source of hypoglycemic agents. The families of plants with the most potent hypoglycemic effects include Leguminoseae (Fabaceae), Lamiaceae, Liliaceae, Cucurbitaceae, Asteraceae, Moraceae, Rosaceae, Euphorbaceae and Araliaceae (30). Biological actions of the plant products used as alternative medicines to treat diabetes are related to their chemical composition. Herbal products or plant products which are rich in phenolic compounds, flavonoids, terpenoids, alkaloids, and other constituents have shown blood glucose lowering activity (28, 30-32). Particularly, schulzeines A, B, and C, radicamines A and B, 2,5-imino-1,2,5-trideoxy-L-glucitol, beta-homofuconojirimycin, myrciacitrin IV, dehydrotrametenolic acid, corosolic acid, 4-(a rhamnopyranosyl) ellagic acid, and 1,2,3,4,6-pentagalloylglucose are among the isolated compounds from plants which have shown significant antidiabetic activities (32).

The antidiabetic activity of medicinal plants depends upon variety of mechanisms. Generally, the mechanisms of action could be grouped as: pancreatic beta cell potassium channel blocking, adrenomimeticism, cAMP (secondary messenger) stimulation; providing certain necessary elements like calcium, zinc, magnesium, manganese and copper for the beta-cells; Inhibition of β-galactocidase and α-glucocidase; preventing oxidative stress that is possibly involved in pancreatic β-cell dysfunction found in diabetes (33); Stimulation of glycogenesis, glycolysis and citric acid cycle and hexose monophosphate shunt; inhibition of gluconeogenesis and glycogenolysis (34-36); improvement in digestion along with reduction in blood sugar and urea; protection of destruction and promotion of regeneration of the β-cells, initiate insulin release; reduction in insulin resistance and/or inhibition in renal glucose reabsorption (37).

The development of metformine was based on the use of Galega officinalis to treat diabetes. Galega officinalis is rich in guanidine which is the hypoglycemic component. Because guanidine is too toxic for clinical use, the alkyl biguanides synthalin A and synthalin B were introduced as oral antidiabetic medications in Europe in the 1920s but were discontinued after insulin became more widely available. The experience with guanidine and biguanides was the base for the development of metformine (38).

The World Health Organization (WHO) has encouraged and recommended the use of herbs as an alternative therapy for diabetes mellitus since medicinal plants are often less expensive, easily accessible, less toxic and suitable to use. Though a wide range of medicinal plants are in use world over, many of them are without valid scientific sanctity. Apparently, a systematic scientific scrutiny of the antidiabetic potentials of these plants has become a matter of utmost importance to justify their application in ethnomedicine (35, 39). Therefore, much effort should be focused on assessing natural products and herbal plants for the discovery of potentially useful alpha-glucosidase inhibitors and aldose reductase inhibitors or other treatment approaches to diabetes (32).

1.3 The experimental plant, *Calpurnia aurea* (Ait.) Benth. (Fabaceae)

The flowering plant family Leguminosae (Fabaceae) contains approximately 650 genera and nearly 20,000 species distributed throughout the world in many ecological settings, from deserts of high latitudes to seasonally dry or wet tropical forests of equatorial regions (40, 41).

The genus *Calpurnia* (Leguminosae) involves seven species which are widely distributed in Africa. *Calpurnia aurea* (Ait.) Benth. (Fabaceae) is a yellow-flowered, multi-stemmed, 3-4 m tall small tree or shrub widely distributed in Africa, ranging from the Cape Province to Eritrea, and it also occurs in southern India (42, 43). Two subspecies of *Calpurnia aurea* are recognized, namely subspecies (subsp.) aureu (occurs in Ethiopia, Zaire, Zimbabwe, Angola, West Africa, and Cape Province) and subsp. Indica which is confined in India (44).

Figure 4: Picture of *Calpurnia aurea* (Ait.) Benth. (By Yaschilal Muche)

Calpurnia aurea from Ethiopia is known locally as “digitta” (Amharic) or “cheka” (Oromiffa). The plant is used in traditional medicine of Ethiopia to treat diverse medical conditions. It is used to treat amoebiasis, giardiasis, malaria, diarrhoea, rabies, diabetes and hypertension (45, 46). The plant has also been used as an insecticide to kill lice (47), to induce uterine contractions (48), and to treat coughs, amoebic dysentery, syphilis, leishmaniasis, tapeworm, trachoma, ringworm,

scabies, elephantiasis, abscesses and wounds as well as stomach ache, vomiting, headache and eye diseases (49-52).

Experimental studies have reported that *Calpurnia aurea* subspecies *aurea* has antimalarial, antihypertensive, antidiarheal, antibacterial, antioxidant and anticancer activities (42, 53-58). The plant has hepatoprotective effect against HAART induced hepatotoxicity, and it can attract and immobilize or kill ticks (43, 59).

Preliminary Phytochemical screening of 80% methanol extract of *Calpurnia aurea* seeds showed the presence of alkaloids, tannins, flavonoids, terpenoids, saponins and phenols (55). The result of preliminary phytochemical screening of 80% methanol extract of *Calpurnia aurea* leafs showed the presence of several secondary metabolites including alkaloids, cardiac glycosides, flavonoids, phenols, phytosteriods, saponins, terpenoids, tannins, proanthocyanidins and flavonols (42, 53, 54).

Different pharmacologically active phytochemicals has been isolated from *C. aurea.* Studies reported the isolation of agglutinins to antigens A and B of human erythrocytes, and the novel quinolizidine alkaloid, calpurnine from the seeds of *Calpurnia aurea* subsp. *aurea*. Subsequent investigations reported several more quinolizidine alkaloids which are characteristic chemotaxonomic markers for the family, Fabaceae (58, 60, 61). Apart from the quinolizidine alkaloids, the flavonoids vicenin-2 (6,8-di-β-D-glucopyranosyl-5,7,4'-trihydroxyflavone), butin (7,3',4'-trihydroxyflavanone) and 3'-hydroxydaidzein (7,3',4' trihydroxyisoflavone) were isolated from the seeds of *C. aurea*, showing flavonoids being the other major class of compounds consistently found in the Fabaceae (62). The stem and bark of *Calpurnia aurea* was investigated phytochemically and yielded a quinolizidine alkaloid, calpurnine which is also found in other parts of the plant. Isoflavones and a pterocarpan were also extracted from the stem and bark of *calpurnia aurea*. Isoflavones are also found in the seeds of *Calpurnia aurea*. Furthermore, the isoflavones isolated from the seeds of *Calpurnia aurea* were shown to have moderate activity against the renal, melanoma and breast cancer cell lines (58).

1.4 Justification of the study

Therapies of DM developed along the principles of western medicine are often limited in efficacy, carry the risk of adverse effects, and are often too costly, especially for the developing world (28). Therefore, treating diabetes mellitus with plant derived compounds which are accessible and do not require laborious pharmaceutical synthesis seems highly attractive.

Calpurnia aurea (Ait.) Benth. (Fabaceae) is traditionally used for the treatment of DM in Ethiopia. An ethnobotanical survey done in shenasha, Agew-awi and Amhara peoples of northwest Ethiopia reported that the leaf as well as the seed of the plant is used orally for the treatment of DM (45). Another survey done in nekemtae town (east wollega, Ethiopia) reported leaf decoction of the plant is taken orally to treat DM (46). However; the antidiabetic activity of this medicinal plant is not scientifically validated.

There is considerable evidence that induction of oxidative stress is a key process in the pathogenesis of DM and diabetic complications (14, 63, 64). The protective effect of antioxidants has been explained in experimental and epidemiological studies, which have demonstrated that antioxidants might be helpful in treating diabetes and its complications through prevention of oxidative stress (13, 14, 64). *Calpurnia aurea* (Ait.) Benth. leaves and seeds have strong *in-vitro* antioxidant activities (42, 57). As a result, this medicinal plant may have antidiabetic activity. Additionally, the antidiabetic activity of medicinal plants is mainly due to the presence of alkaloids, phenolic compounds, flavonoids, terpenoids and other constituents (28, 30, 32, 65, 66). The hydromethanolic extract of both *Calpurnia aurea* seeds and leaves also contain these secondary metabolites known to have blood glucose lowering activity (53-55) so that the plant may have antidiabetic activity.

Accordingly, it is justifiable to conduct a study aimed at the evaluation of the effect of the plant materials on DM and its complication, diabetic dyslipidemia using *in vivo* models. Besides, the finding of this study might serve as baseline information for scientific community to further investigate the plant *Calpurnia aurea* by initiating advanced studies on molecular mechanisms with identification of the specific agent responsible for the antidiabetic effect, which may serve as a lead compound for the development of new antidiabetic drugs.

2 Objectives

2.1 General objective

To evaluate the antidiabetic and antidyslipidemic activities of the hydromethanolic leaf and seed extracts of *Calpurnia aurea* (Ait.) Benth. (Fabaceae) in mice

2.2 Specific objectives

- To carry out acute oral toxicity test of the leaf and seed extracts of *calpurnia aurea* (Ait.) Benth.
- To determine hypoglycemic effect of *calpurnia aurea* leaf and seed extracts in normoglycemic mice
- To determine antihyperglycemic activity of the leaf and seed extracts in oral glucose loaded mice
- To evaluate antihyperglycemic activity of the leaf and seed extracts on streptozotocin-induced diabetic mice
- To examine effect of the leaf and seed extracts of the plant on body weight of streptozotocin-induced diabetic mice
- To determine the effect of the leaf and seed extracts on serum lipid level of streptozotocin-induced diabetic mice

3 Materials and Methods

3.1 Materials

3.1.1 Collection of plant materials

Fresh leaves and matured seeds of *Calpurnia aurea* were collected from Anbessamie (located in south Gondar zone of Amhara region, northwest Ethiopia) in January, 2017. Taxonomic identification of the plant was done by a botanist (Mr. Abyu Eniyew), and a specimen of the plant material was preserved in the Herbarium of biology department, University of Gondar with a voucher number YM001 for future reference.

3.1.2 Drugs, chemicals and instruments

The following drugs, chemicals and instruments were used during the study. Streptozotocin (Sigma Aldrich, Germany), glibenclamide (Julphar pharmaceuticals, Ethiopia), Citric acid monohydrate (Lab tech chemicals, India), Tri-sodium citrate dihydrate (Blulux Labratories, India), Methanol absolute (Nice chemicals, India), 40% glucose solution (Reyoung pharmaceuticals, China), sterilized water for injections (Nirma Ltd., India), Analytical balance, pH meter, i-QARE DS-W® blood glucose meter and strips (Alliance international, Taiwan), Distilled water, automated chemistry analyzer (Shenzhen Mindray Bio-medical Electronics Co., Ltd, China), Whatman filter paper No.1, beakers, funnels, measuring cylinder, glass rod, spatula, pipettes, gavage (oral feeding syringe), animal Cages, Insulin syringe with needle, oven, desiccators. All chemicals used were of analytical grade.

3.2 Methods

3.2.1 Preparation of plant crude extracts

The leaves and seeds of the plant were thoroughly washed with distilled water to remove dirt and then dried under shade with optimal ventilation. Then, the dried leaves and seeds were pulverized separately. The coarse powdered plant materials (900 gram powdered leaves and 1 kg powdered seeds) were macerated in 80% methanol for 72 hours and then the extracts were filtered by using whatman filter paper No.1. The marc was re-macerated two times with fresh solvent, each for 72 hours and the filtrates obtained from the successive maceration were dried in an oven at 40 degree centigrade. Finally, the dried leaf and seed extracts were kept separately in a desiccator until used for the experiment.

3.3 Experimental animals

Healthy Male Swiss albino mice (weighing 25-30 g and age of 8-12 weeks) were used in the study except for the acute oral toxicity tests. The mice were obtained from the Ethiopian public health institute (EPHI) and then they were kept in the animal house of pharmacology department, University of Gondar. The animals were maintained under standard conditions (12 h light and 12 h dark cycle) and allowed free access to standard pellet laboratory diet and water *ad libitum*. Animals were acclimatized to the laboratory conditions for 1 week before the initiation of the experiment.

3.4 Acute toxicity study

Acute oral toxicity tests were done for the leaf and seed extracts of the plant. The tests were done based on the limit and main test recommendations of OECD No 425 Guideline (67). On the first day of the test, one female Swiss albino mouse fasted for 3 hours was given 2000 mg/kg of the extract orally. Then the mouse was kept under strict observation for physical or behavioral changes for 24 h, with special attention during the first 4 hours. Because mortality was not observed in the first mouse which received the leaf extract, other four female mice fasted for 3-4 hours were sequentially administered a single dose of 2000 mg/kg of the leaf extract and then observed in the same manner. The observation was continued for a total of 14 days for any sign of toxicity and mortality. Unlike the leaf extract, mortality was observed in the first mouse that received 2000 mg/kg of the seed extract. Thus, the main test was conducted for the seed extract in order to determine the LD50. In the main test single female mice were dosed in sequence at 48 hour intervals using a starting dose, 175mg/kg and a dose progression factor, 3.2. The dosing was stopped after testing 6 consecutive animals because 5 reversals (response, which is death of mouse, was observed at some dose, and a non-response is observed at the next dose tested) were occurred. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days.

3.5 Grouping and dosing of animals

Male animals were used in all animal models (normoglycemic mice, oral glucose loaded mice, single dose treated diabetic mice, and repeated dose treated diabetic mice) because female mice

are less sensitive to STZ (68, 69), and they are also less sensitive to insulin compared to male animals (70). In the normoglycemic, oral glucose loaded and single dose treated diabetic animal models, mice were randomly divided into eight groups (each group containing 6 mice). In all three animal models; Group I (negative control) was treated with 10ml/kg distilled water (DW); Group II, III and IV were treated with 100 mg/kg, 200 mg/kg and 400 mg/kg hydromethanolic leaf extract respectively; Group V, VI and VII were treated with 2.75 mg/kg, 5.5 mg/kg and 11 mg/kg hydromethanolic seed extract respectively; whereas Group VIII (positive control) was treated with the standard drug, glibenclamide (5 mg/kg). In the repeated dose treated diabetic animal model, mice were randomly divided into nine groups (8 groups of diabetic mice and 1 additional group of normal mice, each group containing 6 mice). Group I (diabetic control) was treated with 10 ml/kg DW; Group II, III and IV (diabetic test groups) were treated with 100 mg/kg, 200 mg/kg and 400 mg/kg hydromethanolic leaf extract respectively; Group V, VI and VII (diabetic test groups) were treated with 2.75 mg/kg, 5.5 mg/kg and 11 mg/kg hydromethanolic seed extract respectively; Group VIII (diabetic positive control group) was treated with the standard drug, 5 mg/kg glibenclamide; whereas Group IX (normal control) was treated with 10 ml/kg DW. Age and body weight of animals were matched across groups in each animal model.

The three doses of each plant extract were determined based on the results of acute oral toxicity studies. Glibenclamide (5 mg/kg) was selected as a standard drug for this study based on reports of earlier studies (71-73). The study was conducted using oral route of administration because people traditionally use the plant materials via the oral route (45, 46). All the doses were administered at a volume not greater than 10ml/kg body weight of mice (67).

3.6 Measurement of blood glucose level

In all cases, blood samples for blood glucose measurement were withdrawn from the tail vein of each animal by cutting the tip of the tail aseptically. BGL was measured using a DS-W® blood glucose meter. Measurement of the blood glucose level was done in triplicate and the average value was taken.

3.7 Induction of experimental diabetes

Diabetes was induced using streptozotocin. The drug was dissolved in 0.1 M cold citrate buffer (pH=4.5). The freshly prepared solution was then administered intraperitonially at 150 mg/kg dose to mice (74) which were fasted overnight for 16 hours prior to administration. Free access to food and water was allowed to the animals thirty minutes after administration of STZ. Six hours after the administration of STZ, animals were allowed to drink 5% glucose solution for the next 24 hours to prevent death secondary to hypoglycemic shock. Four days after STZ injection, animals were screened for diabetes. Mice which showed fasting blood glucose level > 200 mg/dl were included in the study as diabetic mice (72, 75). Immediately after screening, STZ-induced diabetic animals were assigned randomly into different groups to perform the experimental studies. The Bedding of the cages was changed every day after STZ injection to provide dry bedding for polyuric diabetic animals.

In order to prepare 0.1 molar citrate buffer, Citric acid monohydrate (2.101g) and Trisodium citrate dihydrate (2.941g) were accurately weighted and then separately dissolved in 100 ml of sterilized water for injection to prepare 0.1 M citric acid and 0.1 M sodium citrate solutions. After preparation of the solutions, 50 ml of the citric acid solution was mixed with 50 ml sodium citrate solution and then pH was adjusted to 4.5 with 0.1 M sodium citrate solution (76).

3.8 Assessing hypoglycemic activity in normoglycemic mice

Mice, which were fasted overnight for 16 hours, were randomly divided into eight different groups (6 animals per group). Then, the animals were treated according to their respective grouping as mentioned above. Blood glucose level of each mouse was measured just before treatment (at 0 hr) as baseline, and then at 1, 2, 4 and 6 hours post-treatment.

3.9 Evaluating effect of extracts on oral glucose tolerance test

Mice were used for the oral glucose tolerance test as overnight fasting increases insulin sensitivity (insulin-stimulated glucose utilization) specifically in mice (77, 78); thus the model can be sensitive for screening anti-hyperglycemic activity of the plant extracts. Overnight fasted (for 16 hours) mice were randomly divided into 8 groups (6 mice per group). Then, mice were treated with distilled water, hydromethanolic plant extracts and glibenglamide according to their respective grouping. Thirty minutes following each administration (71, 79), 2.5 g/kg of glucose

solution was administered to each animal (71). Blood glucose levels were measured for each animal just before treatment (at 0 minute) as baseline, and then at 30, 60 and 120 minutes following glucose administration (71, 80).

3.10 Assessing antihyperglycemic activity of single dose of the extracts in streptozotocin induced diabetic mice

After overnight fasting for 16 hours, streptozotocin induced diabetic mice were assigned randomly into 8 groups (each group containing 6 diabetic mice). Then, mice were treated with distilled water, hydromethanolic plant extracts and glibenglamide according to their respective grouping. Blood glucose level was measured just before treatment (at 0 hr) as baseline, and then at 2, 4, 6 and 8 hours post-treatment.

3.11 Assessing antihyperglycemic activity, effect on body weight and antidyslipidemic activity of repeated doses of the extracts in streptozotocin-induced diabetic mice

After overnight fasting for 16 hours, STZ-induced diabetic mice and normal mice were randomly assigned into 9 groups (8 groups of diabetic mice and 1 group of normal mice, each group containing 6 mice). Then, mice were treated with distilled water, plant extracts and glibenglamide once daily for 14 days according to their respective grouping as explained above. Blood glucose level and body weight of diabetic mice were measured just before starting treatment on the 1st day of treatment (four days after STZ injection) as baseline, and then on the 7th and 14th day of treatment following overnight fasting for 16 hours (81).

On the 15th day, blood samples were collected in a sterile tube through exsanguination of the overnight fasted (for 16 hours) diabetic mice under diethyl ether anesthesia. The blood samples were left at room temperature for 2 hours to allow coagulation, and then centrifuged at 2000 rpm for 10 minutes. The supernatant was immediately separated from the pellet to prepare serum samples in order to determine the level of triglyceride (TG), total cholesterol (TC), and high density lipoprotein cholesterol (HDL-C) using automated chemistry analyzer.

3.12 Ethical considerations

The experiment was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (82), and the proposal of the study was submitted and approved by the ethical review committee of the school of pharmacy, university of Gondar before the commencement of the study.

3.13 Statistical analysis

The data were expressed as mean ± standard error of the mean. Means of all parameters among groups and within a group were compared using one way ANOVA followed by Tuckey's post hoc test. P-values < 0.05 were considered statistically significant. SPSS Version 20 Software was used for statistical analysis. Percent reduction of blood glucose level was calculated using the formula: $[(G_0 - G_p)/G_0]100$, where G_0 is baseline blood glucose level; and G_p is post treatment blood glucose level.

4 Result

4.1 Percentage yield of plant material extraction

At the end of the extraction process, 154 grams of dried dark-brown gummy leaf extract and 136 grams of dried yellowish brown gummy seed extract were harvested. The percentage yield of the hydromethanolic leaf extract was 17.11 % (w/w); whereas the percentage yield of the seed extract was found to be 13.60 % (w/w).

4.2 Acute oral toxicity study

The Acute toxicity study of *Calpurnia aurea* leaf extract (CALE) didn't show mortality in the animals at the limit dose of 2000 mg/kg during the observation time. Thus, the median lethal dose (LD50) of the leaf extract is greater than 2000 mg/kg. Besides, the toxicity study of CALE did not reveal any signs of toxicity; behavioral, neurological, autonomic or physical changes such as alertness, motor activity, restlessness, convulsions, coma, diarrhea and lacrimation.

Acute oral toxicity study of *Calpurnia aurea* seed extract (CASE) revealed that the seed of the plant is toxic compared to the leaf. It was found that the LD50 of CASE is between 55 mg/kg and 175 mg/kg based on the main test recommendations of OECD guideline no 425 (Table 1).

Table 1: Acute oral toxicity study of the 80 % methanolic seed extract of *Calpurnia aurea*

Test sequence	**Mouse ID**	**Dose (mg/kg)**	**Outcome**	**Remark**
1	1	175	Dead	Convulsion was followed by death within 10 minutes after dosing
2	2	55	Survived	No death and sign of toxicity was observed
3	3	175	Dead	Convulsion was followed by death within 10 minutes after dosing
4	4	55	Survived	No death and sign of toxicity was observed
5	5	175	Dead	Convulsion was followed by death within 10 minutes after dosing
6	6	55	Survived	No death and sign of toxicity was observed

4.3 Hypoglycemic activity of 80% methanolic leaf and seed extracts of *Calpurnia aurea* in normoglycemic mice

The effect of hydromethanolic leaf and seed extracts of *Calpurnia aurea* on fasting blood glucose level of normal mice is summarized in Table 2. Between groups analysis revealed that no significant difference in baseline fasting BGL across groups. CASE 11 mg/kg significantly reduced the BGL at the 4^{th} and 6^{th} hours ($p<0.05$) compared to the negative control. But all the three groups treated with different doses of CALE didn't show a significant reduction in BGL at all time points compared to the negative control group. It was found that BGL was significantly reduced by 5 mg/kg glibenclamide at the 2^{nd} ($p<0.05$), 4^{th} ($p<0.01$) and 6^{th} ($p<0.05$) hour compared to the negative control. Similarly, Comparing GLC treated group with extract treated groups, it was revealed that GLC (5mg/kg) significantly reduced the BGL at the 2^{nd} and 4^{th} hour ($p<0.05$) compared to 100 mg/kg CALE treated group; at the 2^{nd} hr ($p<0.05$) compared to 200 mg/kg CALE treated group; at the 2^{nd} ($p<0.01$), 4^{th} ($p<0.05$) and 6^{th} ($p<0.05$) hour compared to the 400 mg/kg CALE treated group. There was no statistically significant difference in BGL when groups treated with different doses of leaf extract compared with each other at all time points. Similarly, different doses of CASE didn't show statistically significant difference in BGL at all time points when compared with each other. When comparing the CALE treated groups with CASE treated groups, it was found that 11 mg/kg CASE significantly reduced the BGL ($p<0.01$) at the 6^{th} hour compared to 400 mg/kg CALE.

Within group analysis showed that treatment with all the three doses of leaf extract and distilled water didn't significantly reduce the BGL at all time points compared to the respective baseline BGL. It was found that 5.5 mg/kg CASE significantly reduced the BGL at the 6^{th} hr ($p<0.05$) compared to the baseline level with percentage reduction of 20.39%. Similarly, significant reduction in BGL was induced with 11 mg/kg CASE at the 4^{th} ($p<0.01$) and 6^{th} ($p<0.001$) hour compared to the baseline level with percentage reduction in BGL, 32.72% and 46.11% respectively. In addition, the standard dug (glibenclamide) reduced the BGL significantly at the 2^{nd} ($p<0.05$), 4^{th} ($p<0.001$) and 6^{th} ($p<0.001$) hours compared to the baseline level with percentage reduction of 32.37%, 40.63% and 46.61% respectively.

Table 2: Hypoglycemic activity of Hydromethanolic *Calpurnia aurea* leaf and seed extracts in normoglycemic mice

Group	Blood glucose level (mg/dl)				
	0 hr	**1 hr**	**2 hr**	**4 hr**	**6 hr**
DW 10ml/kg	68.17±7.97	70.22±7.90	72.33±8.09	73.5±7.33	68.06±7.99
CALE 100mg/kg	70.67±4.36	73.78±2.76	72.61±3.42	66.33±2.08	64.00±3.88
CALE 200mg/kg	68.83±4.14	73.83±4.08	71.56±4.59	65.56±2.56	56.33±4.92
CALE 400mg/kg	76.72±3.21	79.11±5.96	83.17±3.89	67.17±5.29	67.39±6.61
CASE 2.75mg/kg	69.00±2.52	72.17±9.64	62.56±8.34	60.56±10.17	58.11±10.47
CASE 5.5mg/kg	74.61±3.81	74.00±3.13	72.28±2.41	61.89±1.90	$59.39\pm5.14^{\beta 1}$
CASE 11mg/kg	70.11±3.71	62.50±4.40	59.39±2.73	$47.17\pm3.59^{a1\ \beta 2}$	$37.78\pm3.80^{a1\ d1\ \beta 3}$
GLC 5mg/kg	70.56±5.86	54.00±5.34	$47.72\pm5.03^{a1\ b1\ c1\ d2\ f1\ \beta 1}$	$41.89\pm2.73^{a2\ b1\ d1\ \beta 3}$	$37.67\pm2.4^{a1\ d1\ \beta 3}$

Each value represents mean ± S.E.M, n=6 for each treatment. [a] compared to the negative control, [b] compared to CALE 100mg/kg, [c] compared to CALE 200mg/kg, [d] compared to CALE 400mg/kg, [f] compared to CASE 5.5 mg/kg, [β] compared to baseline blood glucose level. [1]$p < 0.05$, [2]$p < 0.01$ and [3]$p < 0.001$. CALE = *calpurnia aurea* leaf extract, CASE = *calpurnia aurea* seed extract, DW = distilled water, GLC = glibenclamide.

4.4 Antihyperglycemic activity of the hydromethanolic leaf and seed extracts of *Calpurnia aurea* in oral glucose loaded mice

There was no significant difference in baseline BGL across groups just before the administration of DW, extracts and glibenclamide (Table 3). Between groups analysis showed that 5.5 mg/kg and 11 mg/kg CASE significantly reduced the hyperglycemia ($p<0.05$ in both cases) at the 2nd hour compared to the negative control. But, it was found that all doses of CALE didn't show a significant reduction in hyperglycemia at all time points compared to the negative control. Similarly, 5 mg/kg GLC reduced the hyperglycemia significantly at the 1st ($p<0.05$) and 2nd ($p<0.001$) hour post glucose administration compared to the vehicle treated group. Comparing the GLC treated group with plant extract treated groups; 5 mg/kg GLC significantly reduced the hyperglycemia at the 2nd hour ($p<0.05$) compared to 200 mg/kg CALE. There was no statistically significant difference in BGL at all time points when all the six plant extract treated groups compared with each other.

Within a group analysis showed that oral glucose loading caused a statistically significant ($p<0.001$) increment in BGL at 30 minute in all groups compared to the baseline fasting BGL regardless of the treatments given. Additionally, significant hyperglycemia was observed at 1 hr

post glucose load in all groups, except GLC (5 mg/kg) treated group, compared to the respective baseline BGL. But there was no statistically significant difference in BGL at the 2nd hour compared to the baseline level in all groups. Besides, significant reduction in BGL was observed at 60 and 120 minute in all groups including the negative control compared to the respective BGL at 30 minute post glucose load.

Table 3: Effect of *Calpurnia aurea* leaf and seed extracts on oral glucose tolerance in normal mice

Group	Blood glucose level (mg/dl)			
	0 min	**30 min**	**60 min**	**120 min**
DW 10 ml/kg	87.06±8.25	202.95±15.84$^{\beta 3}$	142.17±15.66$^{\beta 1\ \mu 1}$	112.45±13.29$^{\mu 3}$
CALE 100 mg/kg	85.83±8.55	199.50±15.22$^{\beta 3}$	138.06±16.56$^{\beta 1\ \mu 1}$	84.33±6.94$^{\mu 3}$
CALE 200 mg/kg	86.17±5.87	213.00±7.51$^{\beta 3}$	137.00±9.01$^{\beta 2\ \mu 3}$	93.50±5.98$^{\mu 3}$
CALE 400 mg/kg	77.61±7.23	211.72±20.84$^{\beta 3}$	137.61±14.28$^{\beta 1\ \mu 2}$	81.94±4.18$^{\mu 3}$
CASE 2.75 mg/kg	88.67±4.30	210.83±9.65$^{\beta 3}$	132.61±12.07$^{\beta 2\ \mu 3}$	84.28±3.30$^{\mu 3}$
CASE 5.5 mg/kg	84.11±7.65	172.83±13.61$^{\beta 3}$	128.44±11.39$^{\beta 2\ \mu 1}$	80.94±2.40$^{a1\ \mu 3}$
CASE 11 mg/kg	76.50±2.69	202.56±17.12$^{\beta 3}$	130.22±11.38$^{\beta 2\ \mu 3}$	76.45±2.96$^{a1\ \mu 3}$
GLC 5 mg/kg	81.44±1.24	180.72±8.75$^{\beta 3}$	82.83±5.43$^{a1\ \mu 3}$	61.78±8.59$^{a3\ c1\ \mu 3}$

Each value represents mean ± S.E.M, n=6 for each treatment. ^{a} compared to the negative control, ^{c} compared to CALE 200mg/kg, $^{\beta}$ compared to baseline blood glucose level, $^{\mu}$ compared to the blood glucose level at 30 minute. $^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$. CALE = *calpurnia aurea* leaf extract, CASE = *calpurnia aurea* seed extract, DW = distilled water, GLC = glibenclamide. Time refers to time after oral glucose administration.

4.5 Antihyperglycemic activity of single dose of the leaf and seed extracts of *Calpurnia aurea* in streptozotocin-induced diabetic mice

A total of 69 male Swiss albino mice were injected with Streptozotocine and 52 of them were found to be diabetic (fasting BGL > 200 mg/dl) four days after STZ injection, with a success rate of 75.36%. Among the 52 diabetic mice, one died before the administration of the test substances and all the remaining animals survived until the end of the experiment.

Antihyperglycemic activity of single dose of the extracts was studied in STZ induced diabetic mice. As shown in Table 4, between and within group analysis were performed to see BGL differences across the various groups and time points respectively. The between group analysis indicated that no significant difference in baseline fasting BGL across all groups. Similarly, there

was no significant difference in BGL across all groups at the 2nd hour post treatment. Compared to the negative control, a significant BGL reduction was observed at 6th and 8th hour in 5.5 mg/kg and 11 mg/kg CASE treated groups; at 4th, 6th and 8th hour in 5 mg/kg GLC treated group. There was no statistically significant difference in BGL at all time points when groups treated with plant extracts compared to each other, and compared to the positive control.

Within group comparison indicated that significant BGL reduction was not observed in CALE 100mg/kg, CALE 200 mg/kg, CALE 400 mg/kg, CASE 2.75 mg/kg, CASE 5.5 mg/kg and DW treated groups at all time points compared to the baseline fasting BGL. However, percent reduction in BGL was recorded as; 32.58% in CALE 100 mg/kg treated group, 21.59% in CALE 200 mg/kg treated group, 22.75% in CALE 400 mg/kg treated group, 27.47% in CASE 2.75 mg/kg treated group and 50.15% in CASE 5.5 mg/kg treated group at 8th hour compared to the respective baseline fasting BGL. CASE 11 mg/kg was able to decrease the BGL significantly at the 6th and 8th ($p<0.05$) hour compared to the initial value with percentage reduction of 46.36% and 51.47%, respectively. The standard drug, glibenclamide also produced a significant BGL reduction at the 4th, 6th and 8th ($p<0.001$) hour compared to the initial level.

Table 4: Antihyperglycemic activity of single dose leaf and seed extracts of *calpurnia aurea* in STZ-induced diabetic mice

Group	**Blood glucose level (mg/dl)**				
	0 hr	**2 hr**	**4 hr**	**6 hr**	**8 hr**
DW 10 ml/kg	394.11±31.03	383.06±27.65	396.39±26.71	397.45±18.52	399.61±22.00
CALE 100 mg/kg	377.72±43.57	364.39±35.29	288.83±51.29	261.33±54.28	254.67±48.16
CALE 200 mg/kg	314.39±31.99	291.11±38.52	254.78±56.26	250.06±51.84	246.50±56.51
CALE 400 mg/kg	342.67±58.07	313.39±73.93	266.39±53.99	290.00±57.78	264.72±56.88
CASE 2.75 mg/kg	369.45±33.27	307.00±62.98	255.00±49.74	245.50±57.01	235.11±49.68
CASE 5.5 mg/kg	395.39±53.55	308.78±61.45	232.06±60.77	193.83±45.04 ^{a1}	197.11±54.32^{a1}
CASE 11 mg/kg	341.89±50.75	267.06±45.23	194.78±30.46	183.39±27.12$^{a1\ \beta1}$	157.83±19.81$^{a2\ \beta1}$
GLC 5 mg/kg	368.50±43.02	283.39±39.09	176.61±14.01$^{a1\ \beta3}$	171.72±18.27$^{a1\ \beta3}$	155.72±13.59$^{a2\ \beta3}$

Each value represents mean ± S.E.M, n=6 for each treatment. ^{a} compared to the negative control, $^{\beta}$ compared to baseline blood glucose level. ^{1} $p < 0.05$, ^{2} $p < 0.01$, ^{3} $p < 0.001$. CALE = *calpurnia aurea* leaf extract, CASE = *calpurnia aurea* seed extract, DW = distilled water, GLC = glibenclamide.

4.6 Antihyperglycemic activity, effect on body weight and antidyslipidemic activity of the repeated doses of extracts in streptozotocin induced diabetic mice

4.6.1 Antihyperglycemic activity of the repeated doses of leaf and seed extracts of *Calpurnia aurea* in diabetic mice

Between group analysis indicated that no significant difference in baseline fasting BGL across all groups of diabetic mice, but the baseline BGL of the diabetic groups was significantly higher than the baseline BGL of the normal control (Table 5). Groups treated with 2.75 mg/kg, 5.5 mg/kg and 11 mg/kg CASE and 5 mg/kg GLC showed significant reduction in blood glucose level on the 7^{th} and 14^{th} day of treatment compared to the diabetic control. There was no statistically significant difference in BGL at all time points when groups treated with plant extracts compared with each other. Similarly, GLC treated group showed no significant difference in BGL at all time points when compared to plant extract treated groups.

Within group analysis revealed that all the three doses of CASE and the standard drug reduced the BGL significantly at the 7^{th} and 14^{th} day of treatment compared to the baseline level. But groups treated with different doses of CALE, the diabetic control and the normal control didn't show a significant change in BGL at all time points compared to the baseline level.

Table 5: Antihypeglycemic activity of repeated doses of *Calpurnia aurea* leaf and seed extracts in STZ-induced diabetic mice

Group	**Fasting blood glucose level (mg/dl)**			**Percent reduction in baseline BGL**	
	Baseline	**7^{th} day**	**14^{th} day**	**7^{th} day**	**14^{th} day**
Diabetic control	394.11 ± 31.0^{n3}	383.67 ± 45.83^{n3}	387.00 ± 47.77^{n3}	2.6%	1.8%
CALE 100 mg/kg	377.72 ± 43.57^{n3}	251.39 ± 42.39^{n2}	250.44 ± 42.30^{n1}	33.45%	33.69%
CALE 200 mg/kg	314.39 ± 31.99^{n2}	243.56 ± 36.29^{n1}	239.94 ± 35.8^{n1}	22.53%	23.68%
CALE 400 mg/kg	342.67 ± 58.07^{n3}	252.72 ± 29.92^{n2}	250.61 ± 29.57^{n1}	26.25%	26.87%
CASE 2.75 mg/kg	369.45 ± 33.27^{n3}	$222.17\pm34.81^{a1\,\beta1}$	$218.95\pm34.59^{a1\,\beta1}$	39.86%	40.73%
CASE 5.5 mg/kg	395.39 ± 53.55^{n3}	$195.83\pm24.23^{a2\,\beta2}$	$191.17\pm23.91^{a2\,\beta2}$	50.47%	51.65%
CASE 11 mg/kg	341.89 ± 50.75^{n3}	$154.39\pm23.19^{a3\,\beta2}$	$148.22\pm23.22^{a3\,\beta2}$	54.84%	56.65%
GLC 5 mg/kg	368.50 ± 43.02^{n3}	$145.56\pm26.72^{a3\,\beta3}$	$136.67\pm26.41^{a3\,\beta3}$	60.49%	62.91%
Normal control	76.83±2.51	77.67±2.50	78.67±2.75	-1.09%	-2.4%

Each value represents mean ± S.E.M, n=6 for each group. [a] compared to the diabetic control, [n] compared to the normal control. [β] compared to baseline blood glucose level. [1] $p < 0.05$, [2] $p < 0.01$ and [3] $p < 0.001$. CALE = *calpurnia aurea* leaf extract, CASE = *calpurnia aurea* seed extract, GLC = glibenclamide.

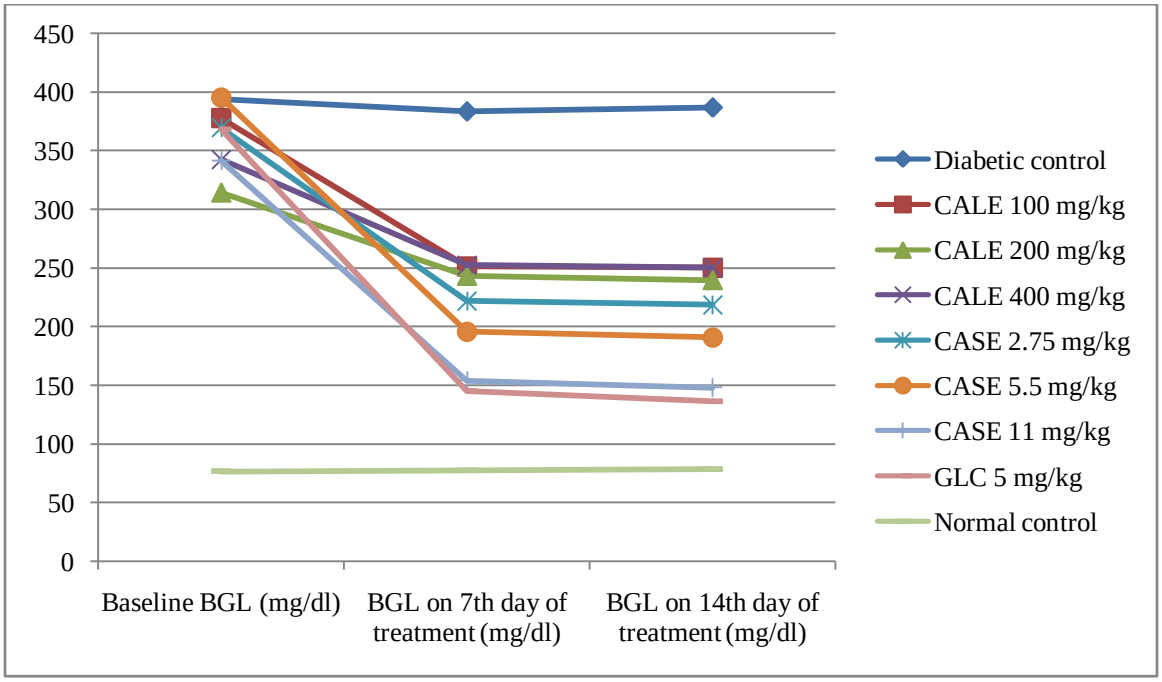

Figure 5: Effect of repeated doses of *Calpurnia aurea* leaf and seed extracts on blood glucose level of diabetic mice. CALE = *calpurnia aurea* leaf extract, CASE = *calpurnia aurea* seed extract, GLC = glibenclamide, BGL = blood glucose level

4.6.2 Effect of the repeated doses of leaf and seed extracts of *Calpurnia aurea* on body weight of diabetic mice

There was no significant difference in body weight of mice across all groups including the normal control just before induction of DM with STZ. STZ produced significant loss of body weight in the diabetic control at the 7th and 14th day of treatment compared to the normal control. The results shown in Table 6 revealed that all the three doses of CASE (2.75, 5.5 and 11 mg/kg) and 5 mg/kg glibenclamide showed significant improvement in body weight at the 14th day of treatment compared to the diabetic control. There was no statistically significant difference in body weight at all time points when groups treated with the plant extracts compared with each other.

Intra-group analysis was done to compare the baseline body weight, which was measured just before starting treatment (four days after STZ administration), with body weight at the 7th and 14th day of treatment. Accordingly, it was found that groups treated with the three doses of CALE ($p<0.05$) and the diabetic control ($p<0.01$) showed significant body weight reduction at the 14th day of treatment compared to the respective baseline body weight.

Table 6: Effect of repeated doses of the leaf and seed extracts of *Calpurnia aurea* on body weight of STZ-induced diabetic mice

Group	**Body weight (g)**			
	Before induction of Diabetes	**Baseline**	**7th day of treatment**	**14th day of treatment**
Diabetic control	28.67±0.95	26.77±0.89	23.88±1.24^{n2}	20.88±1.15$^{n3\,\beta2}$
CALE 100 mg/kg	28.83±1.09	27.98±0.95	25.07±1.06^{n1}	23.78±0.86$^{n3\,\beta1}$
CALE 200 mg/kg	28.83 ±0.91	26.82±1.18	24.83±0.98^{n1}	22.97±0.76$^{n3\,\beta1}$
CALE 400 mg/kg	28.75±0.96	27.70±0.99	25.00±1.02^{n1}	23.25±1.16$^{n3\,\beta1}$
CASE 2.75 mg/kg	28.58±0.99	27.92±1.40	26.93±1.72	27.00±1.75^{a2}
CASE 5.5 mg/kg	28.50±0.75	27.50±0.82	26.68±0.90	26.95±0.89^{a2}
CASE 11 mg/kg	28.92±0.72	27.58±1.01	27.18±1.01	27.67±0.98^{a3}
GLC 5 mg/kg	28.75±0.48	26.07±0.73	25.95±0.71	26.53±0.72^{a2}
Normal control	29.00±0.47	29.45±0.36	30.03±0.53	30.70±0.59

Each value represents mean ± S.E.M, n=6 for each group. ^{a} compared to the diabetic control, ^{n} compared to the normal control. $^{\beta}$ compared to baseline body weight. ^{1} $p < 0.05$, ^{2} $p < 0.01$ and ^{3} $p < 0.001$. CALE = *calpurnia aurea* leaf extract, CASE = *calpurnia aurea* seed extract, GLC = glibenclamide.

4.6.3 Effect of the repeated doses of leaf and seed extracts of *Calpurnia aurea* on serum lipid level of diabetic mice

There was a significant elevation ($p<0.001$) of serum total cholesterol, triglycerides, and significant reduction ($p<0.001$) of HDL cholesterol in the diabetic control mice compared to the normal mice (Table 7). The administration of 5.5 mg/kg and 11 mg/kg *Calpurnia aurea* seed extract for 14 days significantly reduced ($p<0.05$) the levels of serum total cholesterol while significantly increasing ($p<0.05$) the HDL cholesterol level. Similarly, all the three doses of CASE significantly reduced the level of serum triglyceride. The standard drug glibenclamide also significantly reduced ($p<0.001$) the serum cholesterol and triglyceride level while increasing

($p<0.01$) the HDL cholesterol. There was no significant difference in the level of serum TC, TG and HDL-C when groups treated with plant extracts compared with each other.

Table 7: Effect of repeated doses of *Calpurnia aurea* leaf and seed extracts on serum lipid level of diabetic mice

Groups	**Serum lipid level (mg/dl)**		
	TC	**TG**	**HDL-C**
Diabetic control	191.33±4.07^{n3}	164.83±13.49^{n3}	22.17±3.05 ^{n3}
CALE 100mg/kg	168.83±3.67^{n3}	154.00±6.71^{n3}	28.17±2.10
CALE 200 mg/kg	171.67±15.40^{n3}	154.67±6.28^{n3}	29.83±3.91
CALE 400 mg/kg	168.87±11.17^{n3}	148.33±4.68^{n3}	30.50±1.34
CASE 2.75 mg/kg	157.67±10.60^{n3}	128.50±6.21$^{a1\ n3}$	33.00±2.88
CASE 5.5 mg/kg	150.33±10.60$^{a1\ n3}$	127.50±6.73$^{a1\ n3}$	36.50±2.64^{a1}
CASE 11 mg/kg	149.67±4.06$^{a1\ n3}$	119.50±4.22^{a2n2}	37.00±0.58^{a1}
GLC 5 mg/kg	99.50±8.27$^{a3\ b3\ c3\ d3\ e3\ f2\ g2}$	77.50±5.55$^{a3\ b3\ c3\ d3\ e3\ f3\ g2}$	38.50±2.68^{a2}
Normal control	83.83±5.36	73.50±7.26	41.17±4.88

Each value represents mean ± S.E.M, n=6 for each group. **[a]** compared to the diabetic control, **[b]** compared to CALE 100mg/kg, **[c]** compared to CALE 200mg/kg, **[d]** compared to CALE 400mg/kg, **[e]** compared to CASE 2.75mg/kg, **[f]** compared to CASE 5.5mg/kg, **[g]** compared to CASE 11mg/kg, **[n]** compared to the normal control. **[1]** $p < 0.05$, **[2]** $p < 0.01$ and **[3]** $p < 0.001$. CALE = *calpurnia aurea* leaf extract, CASE = *calpurnia aurea* seed extract, GLC = glibenclamide, TC = Total cholesterol, TG = Triglyceride, HDL-C = High density lipoprotein cholesterol.

5 Discussion

Diabetes is one of the largest global health emergencies of the 21st century (5). There is a need for safer and more effective treatment because currently available drug regimens for DM have definite disadvantages. Effective novel compounds with pan-target antidiabetic activity and proven long-term safety should be targeted for patients with coexisting lipid and glucose metabolic disorders. Thus investigating plant derived compounds, which are easily accessible and do not require laborious pharmaceutical synthesis, for the treatment of DM is an important research area (28, 81).

There is no previous study on the acute oral toxicity profile of the 80% methanolic seed extract of *Calpurnia aurea*. But previous acute toxicity tests were done for the leaf extract, although there is discrepancy among the results of the studies (53, 54, 56). This study revealed that unlike the leaf extract which was safe at the dose of 2000 mg/kg, the hydroalcoholic seed extract of *Calpurnia aurea* was found to be relatively toxic with LD50 between 55mg/kg and 175mg/kg. This difference in toxicity might be due to qualitative and quantitative differences in the phytochemical content of the leaf and seed.

In the present study, experimental diabetes in mice was induced using streptozotocin [2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose]. Streptozotocin induced diabetes is a known and well documented model of experimental diabetes in mice (83). Previous studies showed that single interaperitoneal injection of 150 mg/kg STZ can produce sustained hyperglycemia in mice at least for 8 weeks (84). Similarly, the present study revealed that STZ induced persistent hyperglycemia without significant change in BGL during the study period of two weeks as observed in the diabetic control mice. STZ is a better diabetogenic agent than alloxan with wider species effectiveness and greater reproducibility, and this could be attributed to the fact that STZ is more stable in solution before and after injection in animals than alloxan (85). The 3 major mechanisms associated with pancreatic β cell death secondary to STZ exposure are DNA methylation, Nitric oxide and reactive Oxygen species Production (85). STZ toxicity to β cells is short-lived and further impairment of the surviving β cell function is due to hyperglycemic toxicity (86).

In this study, there were no detectable differences in baseline BGL across groups in each animal model; additionally the vehicle treated groups didn't show significant reduction of BGL compared to the baseline level. However, significant reductions in BGL was observed in all models after the administration of the hydroalcoholic seed extract and standard drug, indicating changes induced on blood glucose level were attributed to treatments received.

The study on normoglycemic mice revealed that the 80% methanolic seed extract of *calpurnia aurea* at the dose of 5.5 mg/kg and 11 mg/kg showed significant hypoglycemic activity. Similarly, the seed extract at the dose of 5.5 mg/kg and 11 mg/kg showed significant antihyperglycemic activity after administration of single dose of the extract in oral glucose loaded mice as well as in STZ induced diabetic mice. Additionally, all the three doses of CASE showed significant antihyperglycemic activity and improvement in body weight after administration of repeated doses of the extract in diabetic mice. The hypoglycemic and antihyperglycemic activities of the seed extract were dose dependant. In all cases, higher reduction in BGL was observed with 11mg/kg CASE. The seed extract showed a relatively delayed onset of blood glucose lowering action than the standard drug. This might be due to the presence of compounds with higher glycemic index that could give rise to free glucose after digestion and tend to raise BGL following absorption. The presence of such an effect in the face of the hypoglycemic actions by the active compounds could lead to a delay in the action of the plant extract (72). Unlike the seed extract, the leaf extract of *calpurnia aurea* didn't show a statistically significant blood glucose lowering activity in all animal models, and it didn't significantly improve the body weight of diabetic mice after 14 days of treatment. This suggests the seeds of the plant contain higher amount of the active phytochemicals than the leaves.

The antidiabetic activity of medicinal plants is due to the presence of phytochemicals like alkaloids, phenolic compounds, flavonoids and terpenoids (28, 30, 32, 65, 66). Flavonoids are known to have insulinogenic and pancreatic beta cell regenerating activities (30, 32). Thus, blood glucose lowering effects of the hydroalcoholic extract of *Calpurnia aurea* seeds might be due to the presence of these different secondary metabolites with possible synergistic effects.

It was interesting that antihyperglycemic activity in diabetic mice was observed with glibenclamide which produce its effect via selective blockage of ATP sensitive K^+ channels

(K_{ATP}) in the plasma membrane of β-cells of the pancreas thereby it leads to cytosolic depolarization and release of endogenous insulin (87). This suggests single dose intraperitoneal administration of STZ at a dose of 150 mg/Kg didn't cause complete destruction of β-cells; or few cells remained with the capacity to regenerate and secret insulin.

The blood glucose lowering activity of the hydromethanolic extract of *Calpurnia aurea* seeds may be due to Potentiation of insulin effect either by increasing the secretion of insulin from beta cells of pancreas or by increasing the peripheral glucose uptake (88). However, detailed pharmacological and biochemical researches are required to identify the exact mechanism for the hypoglycemic and antihyperglycemic effects observed in the study.

STZ-induced diabetes is characterized by severe loss in body weight (68, 73). Mice with severe hyperglycemia tend to lose a large percentage of their weight after STZ treatment (68, 84). Similarly, the present study revealed that STZ caused significant body weight loss in the diabetic control mice. The induction of diabetes with STZ leads to loss of body weight due to increased wasting of fat stores (89), muscle and tissue proteins (90, 91). Hence, the weight gain after repeated administration of the hydromethanolic *Calpurnia aurea* seed extract in STZ-induced diabetic mice suggests the antihyperglycemic activity of the extract.

Lipid abnormality is one of the complications of diabetes mellitus, manifested mainly by high serum TG, TC and low HDL-C (21, 73). This lipid abnormality is due to activation of hormone sensitive lipase that leads to increased lipolysis and increased secretion of VLDL from the liver (20, 21). In addition, insulin deficiency causes decreased activity of lipoprotein lipase which leads to decreased clearance of VLDL and chylomicrons (22). In this study, STZ-induced diabetic control mice showed significantly increased serum TG, TC and decreased HDL-cholesterol as expected. Administration of CASE for 2 weeks significantly reduced serum TG, TC, and increased HDL-C in a dose dependant manner, but all the three doses of the leaf extract didn't show significant improvement in diabetic dyslipidemia. It is not known whether CASE had a direct effect on lipid metabolism or the antidyslipidemic activity is achieved only due to the controlled hyperglycemia. But, it can be concluded that CASE improves diabetic dyslipidemia.

6 Conclusion and Recommendation

6.1 Conclusion

In conclusion, this study revealed that the hydromethanolic extract of *Calpurnia aurea* seeds has significant antidiabetic activities justifying the traditional use of the plant for DM. The seed extract also showed improvement in diabetic dyslipidemia and body weight loss. But, the hydromethanolic extract of *Calpurnia aurea* leaves didn't show significant antidiabetic and antidyslipidemic activity, and it didn't improve body weight loss in diabetic animals.

6.2 Recommendation

In future studies, it is important to undergo fractionation and purification to isolate and identify the exact molecules which are responsible for the antidiabetic effect of the plant.

Additionally, sub-acute and chronic toxicity tests should be done to further explain safety issues related to the traditional use of the plant.

The medicinal values of *Calpurnia aurea* should be given further attention with priority to its antidiabetic activities.

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8 Annex

Pictures taken during the Experiment: